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DOI: https://doi.org/10.1515/pteridines.1995.6.3.123

Pteridines
Vol. 6, 1995, pp. 123-125

Short Communication

Characterization of Human Pterin-4a-carbinolamine Dehydratase/Dimerization Cofactor of Hepatocyte Nuclear Factor-1α, and of the Cys81-mutant Involved in Hyperphenylalaninæmia

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(Received June 15, 1995)

Introduction

Human pterin-4a-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor-1α (in the following abbreviated PCD) is a small protein, which has two apparent functions: It assists the phenylalanine hydroxylase (PAH) reaction in that it catalyzes the dehydration of the intermediate pterin-4a-carbinolamine (1), and it has been proposed to induce dimerization of the named factor-1α (2). The amino acid sequence of the protein has been solved (3), and its 3D-structure has been reported (4). Its Cys81Arg mutant has been proposed to cause hyperphenylalaninæmia (5) and to be at the origin of the high content of 7-pterins in the patient. We report here on some properties of PCD which has been expressed in *E. coli* and purified, as well as of two Cys81 mutants (Ser and Arg). Details of the present work have been submitted to EJB for publication (6).

Materials and Methods

A pGEMEX-2-derived vector (Promega) expressing the 104 residues encoding wildtype PCD (wt-PCD) starting with methionine and having six C-terminal histidine residues (His-tag) was generated by PCR-cloning using the human liver cDNA as a template (see Figure 1; (7)). To express the mutant proteins C81S-PCD and C81R-PCD, PCR-directed mutagenesis was performed using specific oligonucleotides with corresponding mismatches (Figure 1). Cloning of PCR products into pGEMEX-2-Nde was performed analogously as for the wild-type PCD. Recombinant expression of wt-PCD, C81S-PCD, and C81R-PCD was achieved in *E. coli* BL21 (DE3) pLysS following a description by Promega. The His-tagged PCD proteins were isolated in a standard one-step affinity purification procedure using a Ni-NTA (nickel-nitrilotriacetic acid) sepharose column (Quiagen).

Results

Details of the expression vectors for wt-PCD, C81S-PCD and C81R-PCD are given in Figure 1. Analysis by ESI-MS of the three affinity purified PCD proteins, and in case of the wt-PCD amino terminal amino acid sequence determination, revealed that they had the N-terminal methionine cleaved off. Thus, these C-terminal poly histidine tagged proteins started with alanine as the first amino acid, similarly to what was found for the mature form of the human (and rat) enzyme (3).

The prominent catalytic properties of PCD and of its two mutants are summarized in the Table.

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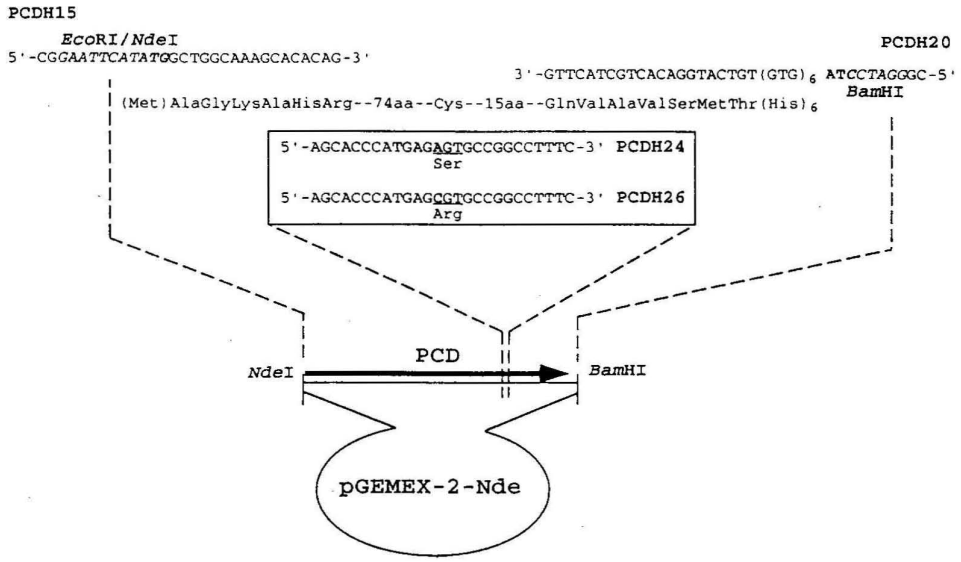

Figure 1. Construction of vectors for expressing wt-PCD, C81S-PCD, and C81R-PCD Histagged proteins. The wt-PCD expression vector was generated by first performing standard PCR amplification with primers PCDH15 and PCDH20 and the human PCD-cDNA as template. Primer PCDH15 contains an *Eco*RI and a *Nde*I site (italics), the ATG start codon (bold) plus the codons for the first 6 residues of the mature form of PCD. The amino terminal methionine is in parenthesis because it was found to be cleaved off following recombinant expression in *E. coli*. Primer PCDH20 encodes the C-terminal seven amino acids of PCD followed by six codons for histidine residues plus a stop codon (bold) and a *Bam*HI restriction site (italics) at its 5'-end. The PCR product encoding the 110 amino acids for the recombinant His-tag PCD was digested with *Nde*I/*Bam*HI endonucleases and ligated into the expression vector pGEMEX-2-Nde (Promega; a second *Nde*I site present in the original vector was removed by site-directed mutagenesis). To generate the vectors for expression of mutant proteins, PCR-directed mutagenesis was performed using primer PCDH24 (for Cys to Ser exchange) or PCDH26 (for Cys to Arg exchange) in combination with primer PCDH20 (see boxed inset; nucleotide exchanges are in bold and relevant codons are underlined). The PCR products were gel purified and used as primers for a second round of PCR in combination with primer PCDH15. Products from these second PCR were cloned into pGEMEX-2-Nde in an analogous way as described above.

They show that PCD and both mutants expressed in *E. coli* are active both using the PAH-linked assay (8) and the synthetic substrate 6,6-dimethyl-pterin-4a-carbinolamine (DCA). Importantly, the activity values are indistinguishable from those obtained using PCD provided to us by Ficner et al (4), which does not carry the His-tag at the C-terminus. This demonstrates that the His-tag does not affect the catalytic properties or binding of our PCD. These recombinant PCD's all bind biopterin at its three oxidation states and also quinonoid 6,6-dimethyl-7,8-dihydropterin, suggesting the presence of a specific recognition site. PCD contains a single Cys81 sulfhydryl residue. Since the corresponding Ser mutant has closely similar properties, a direct role of Cys81 in catalysis can be excluded. Most importantly the Cys81Arg mutant has lower activity and a significantly higher K_m value in the PAH linked assay or using the synthetic substrate DCA compared to wild-type PCD or its Cys81Ser mutant. This is in keeping with the proposal (5) that this mutation is the origin of the reported genetic disorder. On the other hand, when the phenylalanine hydroxylase reaction was carried out using 0.1 µM Cys81Arg-PCD as the stimulant, the production of 7-pterins was effectively suppressed as is the case with wild-type PCD.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft to S.G. (Gh2/7-1) and from the Swiss National Science Foundation (project No. 31-33897.92).

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Table 1. Selected properties of PCD and of its Cys81Ser and Cys81Arg mutants

	wt-PCD	C81S-PCD	C81R-PCD
Vmax PAH-assay (acce–-leration factor)[a]	10	9.5	12
K_m PAH-assay (μM)[a]	0.04	0.05	0.6
V_{max} direct (DCA) assay (nmol/min)[b]	76	52	<20
K_m direct (DCA) assay (μM)[b]	83	80	n.d.[d]
K_d (binding of Pterins)[c] (μM)			
Tetrahydrobiopterin (BH_4)	20	25	20
7,8-Dihydrobiopterin (BH_2)	20	80	20
L-Biopterin	7	100	7
Quinonoid 6,6-dimethyl-7,8-dihydropterin	0.9	20	1

[a] Calculated from the increase in velocity observed upon addition of PCD to the PAH-assay (8). The assay was carried out in 50 mM Tris-HCl, pH 8.3, 20 μg catalase, 0.5 units dihydropteridine reductase, 2.9 nmol BH_4, 100 nmol NADH and 1 μM phenylalanine at 25℃. The activity was determined by observation of the NADH consumption at 340 nm.

[b] Calculated from the dehydration rate of 6,6-dimethyl-pterin-4a-carbinolamine (DCA). The observed rates have been corrected for the spontaneous decay of the DCA. The experiments were carried out in 10 mM Tris-HCl, pH 8.5 at 4℃ with variable concentrations of DCA.

[c] Obtained by titration of the enzyme with pterin and observation of the quenching of the tryptophan fluorescence at 334 nm (λ_{exc}280 nm) according to the method of Rebrin et al.(9). Conditions: 1.1 μM PCD in 100 mM Tris-HCl, pH 8.4. Addition of pterin leads to essentially complete quenching of the tryptophan fluorescence emission.

[d] n.d.; not determined.

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